03/07/02 JC09 Rec'd PCT/PTO

03-08-02 JC20 Rec'd PCT/PTO 07 MAR 2002

FORM PTO-1390 (REV. 10-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 20194P
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 10/070778
INTERNATIONAL APPLICATION NO. PCT/US00/24437	INTERNATIONAL FILING DATE 06 SEPTEMBER 2000	PRIORITY DATE CLAIMED 10 SEPTEMBER 1999

TITLE OF INVENTION
MURF GENE AND ENZYME OF PSEUDOMONAS AERUGINOSA

APPLICANT(S) FOR DO/EO/US
MOHAMED EL-SHERBEINI AND BARBARA AZZOLINA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures [35 U.S.C. 371(f)] at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(l).
4. [ ] A proper Demand for International Preliminary Examination was made and the US was elected by the expiration of the 19th month from the earliest claimed priority date (PCT Article 31).
5. [X] A copy of the International Application as filed [35 U.S.C. 371(c)(2)].
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed [35 U.S.C. 371(c)(2)].
7. [ ] Amendments to the claims of the International Application under PCT Article 19 [35 U.S.C. 371(c)(3)].
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)].
9. [X] An oath or declaration of the inventor(s) [35 U.S.C. 371(c)(4)].
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 [35 U.S.C. 371(c)(5)].

Items 11 to 16 below concern other document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A **FIRST** preliminary amendment.
    [ ] A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

EXPRESS MAIL CERTIFICATE
DATE OF DEPOSIT: March 7, 2002
EXPRESS MAIL NO.: EL523906940US
I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS EXPRESS MAIL "POST OFFICE TO ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.
MAILED BY: Christine Cuffe
DATE: 3-7-02

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070778	PCT/US00/24437	20194P

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE [37 CFR 1.492(a)(1)-(5)]:		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee [37 CFR 1.445(a)(2)] paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1,040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee [37 CFR 1.445(a)(2)] paid to USPTO..... **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$100.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date [37 CFR 1.492(e)].	$0.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	14 - 20 =	0	X **$18.00**	$0.00	
Independent Claims	4 - 3 =	1	X **$84.00**	$84.00	
Multiple dependent claim(s) (if applicable)			+ **$280.00**	$0.00	
TOTAL OF ABOVE CALCULATIONS =				$184.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.					
SUBTOTAL =				$184.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date [37 CFR 1.492(f)]. +				$0.00	
TOTAL NATIONAL FEE =				$184.00	
Fee for recording the enclosed assignment [37 CFR 1.21(h)]. The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +					
TOTAL FEES ENCLOSED =				$184.00	
				Amount to be refunded	
				charged	

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 13-2755 in the amount of $184.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to the Deposit Account No. 13-2755. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive [37 CFR 1.137(a) or (b)] must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

MERCK & CO., INC.
Patent Department, RY60-30
P.O. Box 2000
126 East Lincoln Avenue
Rahway, New Jersey 07065-0970

DATE: March 07, 2002

PHONE #: (732) 594-4678

SIGNATURE

MICHAEL D. YABLONSKY
NAME

40,407
REGISTRATION NUMBER

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	El-Sherbeini, et al.	
Serial No.:	TO BE ASSIGNED – Case No. 20194P	Art Unit:
Filed:	HEREWITH	Examiner:
For:	MURF GENE AND ENZYME OF PSEUDOMONAS AERUGINOSA	

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Applicant requests entry of the amendments presented herein. Applicant also requests examination of the application in view of the amendments presented herein. No fee is believed to be due in connection with the filing of this Preliminary Amendment. However, the Commissioner is authorized to charge Deposit Account 13-2755 for the Petition fee and any other fee(s) required to effect this Petition.

EXPRESS MAIL CERTIFICATE
DATE OF DEPOSIT March 7 2002
EXPRESS MAIL NO. EL523906940US
I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS EXPRESS MAIL "POST OFFICE TO ADDRESSEE" BEFORE 5 P.M. ON THE ABOVE DATE IN AN ENVELOPE ADDRESSED TO ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C., 20231.
MAILED BY Christa Cuffe
DATE 3-7-02

PLEASE AMEND THE SPECIFICATION AS FOLLOWS:

In the Specification:

--This application claims the benefits of U. S. Provisional Application Serial Number 60/153,293, filed on September 10, 1999 and is a National Stage Filing of PCT/US00/24437, having an International Filing Date of September 06, 2000.--

In the Claims:

Please cancel Claims 12, 13 and 14.

Please replace Claim 1 with the clean version presented below:

1. (AMENDED) A purified and isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(b) a polynucleotide which is complementary to the polynucleotide of (a),

(c) a polynucleotide that hybridizes with a polynucleotide of (a) or (b) under stringent conditions.

Please replace Claim 8 with the clean version presented below:

8. (AMENDED) A purified and isolated polypeptide having an amino acid sequence of SEQ ID NO: 2.

Please replace Claim 9 with the clean version presented below:

9. (AMENDED) A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing at least one host cell harboring an expression vector that includes a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2, and

(b) contacting at least one of said cells with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the polypeptide in the presence of the candidate.

Please replace Claim 15 with the clean version presented below:

15. (AMENDED) A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing a sample that includes a MurF polypeptide having an amino acid sequence of SEQ ID NO: 2, and

(b) contacting said sample with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the MurF polypeptide in the presence of the candidate.

CONDITIONAL PETITION

Applicant hereby makes a Conditional Petition for any relief available to correct any defect in connection with this filing, or any defect remaining in this application after this filing. The Commissioner is authorized to charge deposit account 13-2755 for the petition fee and any other fee(s) required to effect this Conditional Petition.

Respectfully submitted,

By_____

Michael D. Yablonsky, Ph.D.
Reg. No. 40,407
Attorney for Applicant

MERCK & CO., INC.
P.O. Box 2000
Rahway, New Jersey 07065-0907
(732) 594-4678

Date: March 7, 2002

Appendix Showing Amendments to the Claims

1. (AMENDED) A purified and isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(b) a polynucleotide which is complementary to the polynucleotide of (a),

(c) [a polynucleotide representing a naturally occurring mutant or polymorphic form of (a),

(d)]a polynucleotide that hybridizes with a polynucleotide of (a)[,] or (b)[, or (c)] under stringent conditions[, and

(e) a polynucleotide comprising at least 25 nucleotides of the polynucleotide of (a), (b) or (c), said 25 nucleotides being specific for *murF* gene of *Pseudomonas aeruginosa*].

8. (AMENDED) A purified and isolated polypeptide having an amino acid sequence [selected from the group consisting of

(a) a polypeptide having an amino acid sequence] of SEQ ID NO:2[,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a)].

9. (AMENDED) A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing at least one host cell harboring an expression vector that includes a polynucleotide [selected from the group consisting of:

(i) a polynucleotide] encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2[.

(ii) a polynucleotide which is complementary to the polynucleotide of (i),

(iii) a polynucleotide representing a naturally occurring mutant or polymorphic form of (i)], and

(b) contacting at least one of said cells with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the polypeptide in the presence of the candidate.

15. (AMENDED) A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing a sample that includes a MurF polypeptide [selected from the group consisting of:

(i) a polypeptide] having an amino acid sequence of SEQ ID NO: 2[.

(ii) a polypeptide that is a functional derivative of the polypeptide of (i),

(iii) a polypeptide representing a naturally occurring mutant or polymorphic form of (i)], and

(b) contacting said sample with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the MurF polypeptide in the presence of the candidate.

TITLE OF THE INVENTION

MURF GENE AND ENZYME OF *PSEUDOMONAS AERUGINOSA*

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/153,293, filed September 10, 1999, the contents of which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

This invention relates to the genes and enzymes involved in cell wall synthesis in bacteria, and particularly to the inhibition of such enzymes.

BACKGROUND OF THE INVENTION

The pathway of peptidoglycan biosynthesis is both essential and unique to bacteria, making it an attractive target for antibiotic research. Several enzymes in this pathway are molecular targets of naturally occurring antibiotics such as fosfomycin, cycloserine, b-lactams and vancomycin

The construction of the peptidoglycan begins in the cytoplasm with an activated sugar molecule, UDP-N-acetylglucosamine. After two reactions (catalyzed by MurA and MurB) that result in the placement of a lactyl group on the 3-OH of the glucosamine moiety, a series of ATP-dependent amino acid ligases (MurC, -D, -E, and -F) catalyze the stepwise synthesis of the pentapeptide sidechain using the newly synthesized lactyl carboxylate as the first acceptor site. After attachment of the sugar pentapeptide to a lipid carrier in the plasma membrane, another glucosamine unit is added to the 4-OH of the muramic acid moiety. The completed monomeric building block is moved across the membrane into the periplasm where the penicillin-binding proteins enzymatically add it into the growing cell wall (Lugtenberg, E. J. J., 1972, Studies on *Escherichia coli* enzymes involved in the synthesis of Uridine Diphosphate-N-Acetyl-Muramyl-pentapeptide. J. Bacteriol. 110:26-34; Mengin-

Lecreulx, D., B. Flouret, and J. van Heijenoort, 1982, Cytoplasmic steps of peptidoglycan synthesis in *Escherichia coli*. J. Bacteriol. 151: 1109-1117).

Because the pentapeptide sidechain is not synthesized ribosomally it contains more diverse chemical functionality than a typical peptide, both structurally and stereochemically. Two of the enzymes catalyze the addition of D-amino acids (MurD and MurF) and MurE mediates the formation of a peptide bond between the g-carboxylate of D-glutamate and the amino group of L-lysine. Presumably these structures render the exposed peptidoglycan resistant to the action of proteases, but they also imply that the active sites of the enzymes must have unusual structures in order to handle the somewhat uncommon substrates. These unusual active sites are targets to bind novel inhibitors that can have antimicrobial activity.

Although peptidoglycan assembly is a proven target for antibiotics, there are no known inhibitors for many of the enzymes of the pathway. Since these enzymes are conserved among eubacteria, inhibitors of this pathway are likely to be broad spectrum antibiotics. Among these potential enzyme targets is MurF, UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap : D-alanine-D-alanine ligase. This enzyme is a target for the antibiotic cycloserine (Kleinkauf H and H. von Dohren. 1990. Nonribosomal biosynthesis of peptide antibiotics. Eur J Biochem. 192:1-15). This validates the assumption that inhibitors of this enzyme are likely to lead to antibiotics for treating infections with either Gram (-ve) or Gram (+ve) bacteria.

SUMMARY OF THE INVENTION

Polynucleotides and polypeptides of *Pseudomonas aeruginosa* MurF, an enzyme involved in bacterial cell wall biosynthesis are provided. The recombinant MurF enzyme is catalytically active in ATP-dependent D-alanine-D-alanine addition reactions. The enzyme is used in *in vitro* assays to screen for antibacterial compounds that target cell wall biosynthesis. The invention includes the polynucleotides, proteins encoded by the polynucleotides, and host cells expressing the recombinant enzyme, probes and primers, and the use of these molecules in assays.

An aspect of this invention is a polynucleotide having a sequence encoding a *Pseudomonas aeruginosa* MurF protein, or a complementary sequence. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In other embodiments, the encoded protein can be a naturally occurring mutant or polymorphic form of the protein. In preferred embodiments the polynucleotide can

be DNA, RNA or a mixture of both, and can be single or double stranded. In particular embodiments, the polynucelotide is comprised of natural, non-natural or modified nucleotides. In some embodiments, the internucleotide linkages are linkages that occur in nature. In other embodiments, the internucleotide linkages can be non-natural linkages or a mixture of natural and non-natural linkages. In a most preferred embodiment, the polynucleotide has a sequence shown in SEQ ID NO:1.

An aspect of this invention is a polynucleotide having a sequence of at least about 25 contiguous nucleotides that is specific for a naturally occurring polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein. In particular preferred embodiments, the polynucleotides of this aspect are useful as probes for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein. In other particular embodiments, the polynucleotides of this aspect are useful as primers for use in nucleic acid amplification based assays for the specific detection of the presence of a polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein. In preferred embodiments, the polynucleotides of this aspect can have additional components including, but not limited to, compounds, isotopes, proteins or sequences for the detection of the probe or primer.

An aspect of this invention is an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In particular embodiments, the vector can have any of a variety of regulatory regions known and used in the art as appropriate for the types of host cells the vector can be used in. In a most preferred embodiment, the vector has regulatory regions appropriate for the expression of the encoded protein in gram-negative prokaryotic host cells. In other embodiments, the vector has regulatory regions appropriate for expression of the encoded protein in gram-positive host cells, yeasts, cyanobacteria or actinomycetes. In some preferred embodiments the regulatory regions provide for inducible expression while in other preferred embodiments the regulatory regions provide for constitutive expression. Finally, according to this aspect, the expression vector can be derived from a plasmid, phage, virus or a combination thereof.

An aspect of this invention is host cell comprising an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein, or a complementary sequence, and regulatory regions. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2. In preferred

embodiments, the host cell is a yeast, gram-positive bacterium, cyanobacterium or actinomycete. In a most preferred embodiment, the host cell is a gram-negative bacterium.

An aspect of this invention is a process for expressing a MurF protein of *P. aeruginosa* in a host cell. In this aspect a host cell is transformed or transfected with an expression vector including a polynucleotide encoding a *Pseudomonas aeruginosa* MurF protein, or a complementary sequence. According to this aspect, the host cell is cultured under conditions conducive to the expression of the encoded MurF protein. In particular embodiments the expression is inducible or constitutive. In a particular embodiment the encoded protein has a sequence corresponding to SEQ ID NO:2.

An aspect of this invention is a purified polypeptide having an amino acid sequence of SEQ ID NO:2 or the sequence of a naturally occurring mutant or polymorphic form of the protein.

An aspect of this invention is a method of determining whether a candidate compound can inhibit the activity of a *P. aeruginosa* MurF polypeptide. According to this aspect a polynucleotide encoding the polypeptide is used to construct an expression vector appropriate for a particular host cell. The host cell is transformed or transfected with the expression vector and cultured under conditions conducive to the expression of the MurF polypeptide. The cell is contacted with the candidate. Finally, one measures the activity of the MurF polypeptide in the presence of the candidate. If the activity is lower relative to the activity of the protein in the absence of the candidate, then the candidate is a inhibitor of the MurF polypeptide. In preferred embodiments, the polynucleotide encodes a protein having an amino acid sequence of SEQ ID NO:2 or a naturally occurring mutant of polymorphic form thereof. In other preferred embodiments, the polynucleotide has the sequence of SEQ ID NO:1. In particular embodiments, the relative activity of MurF is determined by comparing the activity of the MurF in a host cell. In some embodiments, the host cell is disrupted and the candidate is contacted to the released cytosol. In other embodiments, the cells can be disrupted contacting with the candidate and before determining the activity of the MurF protein. Finally, according to this aspect the relative activity can determined by comparison to a previously measured or expected activity value for the MurF activity in the host under the conditions. However, in preferred embodiments, the relative activity is determined by measuring the activity of the MurF in a control cell that was not contacted with a candidate compound. In

particular embodiments, the host cell is a pseudomonad and the protein inhibited is the MurF produced by the pseudomonad.

An aspect of this invention is a compound that is an inhibitor of a *P. aeruginosa* MurF protein an assay described herein. In preferred embodiments, the compound is an inhibitor of a *P. aeruginosa* MurF protein produced by a host cell comprising an expression vector of this invention. In most preferred embodiments, the compound is also an inhibitor of MurF protein produced by a pathogenic strain *P. aeruginosa* and also inhibits the growth of said pseudomonad.

An aspect of this invention is a pharmaceutical preparation that includes an inhibitor of *P. aeruginosa* MurF and a pharmaceutically acceptable carrier.

An aspect of this invention is a method of treatment comprising administering a inhibitor of the *P. aeruginosa* MurF to a patient. The treatment can be prophylactic or therapeutic. In preferred embodiments, the appropriate dosage for a particular patient is determined by a physician.

By "about" it is meant within approximately 10-20% greater or lesser than particularly stated.

As used herein an "inhibitor" is a compound that interacts with and inhibits or prevents a polypeptide of MurF from catalyzing the ATP-dependent addition of D-alanine-D-alanine to an m-Dap residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap.

As used herein a "modulator" is a compound that interacts with an aspect of cellular biochemistry to effect an increase or decrease in the amount of a polypeptide of MurF present in, at the surface or in the periplasm of a cell, or in the surrounding serum or media. The change in amount of the MurF polypeptide can be mediated by the effect of a modulator on the expression of the protein, *e.g.*, the transcription, translation, post-translational processing, translocation or folding of the protein, or by affecting a component(s) of cellular biochemistry that directly or indirectly participates in the expression of the protein. Alternatively, a modulator can act by accelerating or decelerating the turnover of the protein either by direct interaction with the protein or by interacting with another component(s) of cellular biochemistry which directly or indirectly effects the change.

All of the references cited herein are incorporated by reference in their entirety as background material.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A & 1B. Nucleotide sequence (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO:2) of *P. aeruginosa murF*. The amino acid sequence (SEQ ID NO:2) is presented in three-letter code below the nucleotide sequence (nucleotides 57 to 1431 of SEQ ID NO: 1).

FIG. 2. Production of MurF Protein. Lane 1, Molecular weight markers; Lane2, IPTG-induced lysate of cells (BL21(DE3)/pLysS) containing the control vector pET-15b; Lane 3, uninduced cell lysate containing the control vector pET-15b; lane 4, column-purified MurF; Lane 5 IPTG-induced lysate of cells expressing MurF; Lane 6, uninduced lysate of cells containing *murF*.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides polynucleotides and polypeptides of a cell wall biosynthesis gene from *Pseudomonas aeruginosa*, referred to herein as MurF. The polynucleotides and polypeptides are used to further provide expression vectors, host cells comprising the vectors, probes and primers, antibodies against the MurF protein and polypeptides thereof, assays for the presence or expression of MurF and assays for the identification of modulators and inhibitors of MurF.

Bacterial MurF, UDP-N-acetylmuramyl-L-alanine-D-glutamate-m-Dap:D-alanine-D-alanine ligase, a cytoplasmic peptidoglycan biosynthetic enzyme, catalyzes the ATP-dependent addition of D-alanine-D-alanine to the m-Dap residue of the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap precursor generating the pentapeptide UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap-D-alanine-D-alanine.

The *murF* gene was cloned from *Pseudomonas aeruginosa*. Sequence analysis of the *P. aeruginosa murF* gene revealed an open reading frame of 459 amino acids. The deduced amino acid sequence of *P. aeruginosa* MurF is homologous to MurF from *Escherichia col, Bacillus subtilis* and other bacteria . Recombinant MurF protein from *P. aeruginosa* was over-produced as His-tagged fusion protein in *Escherichia coli* host cells and the enzyme was purified to apparent homogeneity. The recombinant enzyme catalyzed the ATP-dependent addition of D-alanine-D-alanine to the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap precursor.

Nucleic acids encoding *murF* from *Pseudomonas aeruginosa* are useful in the expression and production of the *P. aeruginosa* MurF protein. The

nucleic acids are also useful in providing probes for detecting the presence of *P. aeruginosa murF*.

Polynucleotides

Polynucleotides useful in the present invention include those described herein and those that one of skill in the art will be able to derive therefrom following the teachings of this specification. A preferred aspect of the present invention is an isolated nucleic acid encoding a MurF protein of *Pseudomonas aeruginosa*. A preferred embodiment is a nucleic acid having the sequence disclosed in FIG. 1, SEQ ID NO:1 and disclosed as follows:

TCCGTTCTCC GACATCGAGC AGGCCGAGCG CGCCCTGGCC GCCTGGGAGG
TGCCGCATGC TTGAGCCTCT TCGCCTCAGC CAGTTGACGG TCGCGCTGGA
CGCCCGCCTG ATCGGCGAGG ACGCCGTCTT TTCGGCGGTT TCCACCGACA
GTCGCGCCAT CGGGCCCGGC CAACTGTTCA TTGCCCTGAG TGGGCCGCGT
TTCGACGGCC ACGACTATCT CGCCGAGGTT GCCGCCAAGG GCGCGGTGGC
TGCGCTGGTG GAGCGCGAAG TCGCCGACGC GCCCTTGCCG CAATTGCTGG
TGCGCGATAC CCGTGCGGCC CTGGGGCGAC TGGGCGCGCT GAACCGGCGC
AAGTTCACCG GCCCGCTGGC GGCCATGACG GGCTCCAGCG GCAAGACCGC
GGTCAAGGAG ATGCTCGCCA GCATCCTGCG TACCCAGGCC GGCGATGCCG
AGTCGGTGCT GGCTACCCGT GGCAATCTGA ACAACGACCT CGGCGTACCG
CTGACCCTGC TGCAACTGGC GCCGCAGCAC CGTAGCGCAG TGATCGAACT
GGGCGCCTCG CGCATCGGCG AGATCGCCTA CACGGTCGAG CTGACCCGCC
CGCACGTGGC GATCATCACC AATGCCGGAA CCGCCCATGT CGGCGAGTTC
GGCGGACCGG AGAAGATCGT CGAGGCGAAG GGCGAGATAC TCGAAGGGCT
GGCCGCCGAC GGCACCGCCG TACTGAACCT GGACGACAAG GCCTTCGACA
CCTGGAAGGC CCGTGCCAGC GGCCGTCCGT TGCTGACTTT CTCCCTCGAC
CGGCCCCAGG CCGATTTCCG CGCCGCCGAT CTGCAGCGCG ATGCGCGCGG
CTGCATGGGC TTCAGGCTGC AGGGCGTAGC GGGTGAAGCG CAGGTCCAGC
TCAACCTGCT GGGGCGGCAC AATGTCGCCA ATGCCCTGGC TGCGGCCGCT
GCCGCCCATG CACTGGGCGT GCCGCTGGAT GGGATCGTCG CCGGGCTGCA
GGCGCTGCAG CCGGTCAAGG GCCGCGCGGT AGCGCAACTG ACCGCCAGCG
GGCTGCGTGT GATAGACGAC AGCTACAACG CCAACCCCGC GTCAATGCTG
GCGGCGATTG ATATACTGAG CGGCTTTTCC GGGCGCACCG TCCTGGTCCT
CGGAGACATG GGCGAACTCG GTTCCTGGGC CGAGCAGGCC CACCGCGAGG

TGGGCGCCTA CGCCGCTGGC AAGGTGTCCG CGCTCTATGC GGTCGGACCG
CTGATGGCCC ACGCCGTACA GGCGTTCGGC GCCACGGGCC GGCACTTCGC
CGACCAGGCC AGCCTGATCG GGGCGCTGGC CACCGAACAA CCGACAACCA
CCATTTTGAT CAAGGGTTCC CGCAGTGCGG CGATGGACAA AGTCGTCGCG
GCGCTGTGCG GTTCCTCCGA GGAGAGTCAC TAATGCTCCT GCTGCTGGC (SEQ ID NO:1)

The translation initiation and termination codons are underlined.

The isolated nucleic acid molecule of the present invention can include a ribonucleic or deoxyribonucleic acid molecule, which can be single (coding or noncoding strand) or double stranded, as well as synthetic nucleic acid, such as a synthesized, single stranded polynucleotide.

The present invention also relates to recombinant vectors and recombinant hosts, both prokaryotic and eukaryotic, which contain the substantially purified nucleic acid molecules disclosed throughout this specification.

As used herein a "polynucleotide" is a nucleic acid of more than one nucleotide. A polynucleotide can be made up of multiple polynucleotide units that are referred to by description of the unit. For example, a polynucleotide can comprise within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is a regulatory region(s) and/or other polynucleotide units commonly used in the art.

An "expression vector" is a polynucleotide having regulatory regions operably linked to a coding region such that, when in a host cell, the regulatory regions can direct the expression of the coding sequence. The use of expression vectors is well known in the art. Expression vectors can be used in a variety of host cells and, therefore, the regulatory regions are preferably chosen as appropriate for the particular host cell.

A "regulatory region" is a polynucleotide that can promote or enhance the initiation or termination of transcription or translation of a coding sequence. A regulatory region includes a sequence that is recognized by the RNA polymerase, ribosome, or associated transcription or translation initiation or termination factors of a host cell. Regulatory regions that direct the initiation of transcription or translation can direct constitutive or inducible expression of a coding sequence.

Polynucleotides of this invention contain full length or partial length sequences of the MurF gene sequences disclosed herein. Polynucleotides of this invention can be single or double stranded. If single stranded, the polynucleotides can

be a coding, "sense," strand or a complementary, "antisense," strand. Antisense strands can be useful as modulators of the gene by interacting with RNA encoding the MurF protein. Antisense strands are preferably less than full length strands having sequences unique or specific for RNA encoding the protein.

The polynucleotides can include deoxyribonucleotides, ribonucleotides or mixtures of both. The polynucleotides can be produced by cells, in cell-free biochemical reactions or through chemical synthesis. Non-natural or modified nucleotides, including inosine, methyl-cytosine, deaza-guanosine, etc., can be present. Natural phosphodiester internucleotide linkages can be appropriate. However, polynucleotides can have non-natural linkages between the nucleotides. Non-natural linkages are well known in the art and include, without limitation, methylphosphonates, phosphorothioates, phosphorodithionates, phosphoroamidites and phosphate ester linkages. Dephospho-linkages are also known, as bridges between nucleotides. Examples of these include siloxane, carbonate, carboxymethyl ester, acetamidate, carbamate, and thioether bridges. "Plastic DNA," having, for example, N-vinyl, methacryloxyethyl, methacrylamide or ethyleneimine internucleotide linkages, can be used. "Peptide Nucleic Acid" (PNA) is also useful and resists degradation by nucleases. These linkages can be mixed in a polynucleotide.

As used herein, "purified" and "isolated" are utilized interchangeably to stand for the proposition that the polynucleotide, protein and polypeptide, or respective fragments thereof in question have been removed from the *in vivo* environment so that they exist in a form or purity not found in nature. Purified or isolated nucleic acid molecules can be manipulated by the skilled artisan, such as but not limited to sequencing, restriction digestion, site-directed mutagenesis, and subcloning into expression vectors for a nucleic acid fragment as well as obtaining the wholly or partially purified protein or protein fragment so as to afford the opportunity to generate polyclonal antibodies, monoclonal antibodies, or perform amino acid sequencing or peptide digestion. Therefore, the nucleic acids claimed herein can be present in whole cells or in cell lysates or in a partially or substantially purified form. It is preferred that the molecule be present at a concentration at least about five-fold to ten-fold higher than that found in nature. A polynucleotide is considered substantially pure if it is obtained purified from cellular components by standard methods at a concentration of at least about 100-fold higher than that found in nature. A polynucleotide is considered essentially pure if it is obtained at a concentration of at

least about 1000-fold higher than that found in nature. We most prefer polynucleotides that have been purified to homogeneity, that is, at least 10,000 - 100,000 fold. A chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors by the standards stated above.

Included in the present invention are assays that employ further novel polynucleotides that hybridize to *P.aeruginosa murf* sequences under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization step carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, *et al.*, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

Polypeptides

A preferred aspect of the present invention is a substantially purified form of the MurF protein from *Pseudomonas aeruginosa*. A preferred embodiment is a protein that has the amino acid sequence which is shown in FIG. 1, in SEQ ID NO:2 and disclosed as follows:

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MetLeuGluProLeuArgLeuSerGlnLeuThrValAlaLeuAspAlaArgLeuIleGly
GluAspAlaValPheSerAlaValSerThrAspSerArgAlaIleGlyProGlyGlnLeu
PheIleAlaLeuSerGlyProArgPheAspGlyHisAspTyrLeuAlaGluValAlaAla
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LysGlyAlaValAlaAlaLeuValGluArgGluValAlaAspAlaProLeuProGlnLeu
LeuValArgAspThrArgAlaAlaLeuGlyArgLeuGlyAlaLeuAsnArgArgLysPhe
ThrGlyProLeuAlaAlaMetThrGlySerSerGlyLysThrAlaValLysGluMetLeu
AlaSerIleLeuArgThrGlnAlaGlyAspAlaGluSerValLeuAlaThrArgGlyAsn
LeuAsnAsnAspLeuGlyValProLeuThrLeuLeuGlnLeuAlaProGlnHisArgSer
AlaValIleGluLeuGlyAlaSerArgIleGlyGluIleAlaTyrThrValGluLeuThr
ArgProHisValAlaIleIleThrAsnAlaGlyThrAlaHisValGlyGluPheGlyGly
ProGluLysIleValGluAlaLysGlyGluIleLeuGluGlyLeuAlaAlaAspGlyThr
AlaValLeuAsnLeuAspAspLysAlaPheAspThrTrpLysAlaArgAlaSerGlyArg
ProLeuLeuThrPheSerLeuAspArgProGlnAlaAspPheArgAlaAlaAspLeuGln
ArgAspAlaArgGlyCysMetGlyPheArgLeuGlnGlyValAlaGlyGluAlaGlnVal
GlnLeuAsnLeuLeuGlyArgHisAsnValAlaAsnAlaLeuAlaAlaAlaAlaAlaAla
HisAlaLeuGlyValProLeuAspGlyIleValAlaGlyLeuGlnAlaLeuGlnProVal
LysGlyArgAlaValAlaGlnLeuThrAlaSerGlyLeuArgValIleAspAspSerTyr
AsnAlaAsnProAlaSerMetLeuAlaAlaIleAspIleLeuSerGlyPheSerGlyArg
ThrValLeuValLeuGlyAspMetGlyGluLeuGlySerTrpAlaGluGlnAlaHisArg
GluValGlyAlaTyrAlaAlaGlyLysValSerAlaLeuTyrAlaValGlyProLeuMet
AlaHisAlaValGlnAlaPheGlyAlaThrGlyArgHisPheAlaAspGlnAlaSerLeu
IleGlyAlaLeuAlaThrGluGlnProThrThrThrIleLeuIleLysGlySerArgSer
AlaAlaMetAspLysValValAlaAlaLeuCysGlySerSerGluGluSerHis (SEQ ID NO:2)

The present invention also relates to biologically active fragments and mutant or polymorphic forms of MurF polypeptide sequence as set forth as SEQ ID NO: 2, including but not limited to amino acid substitutions, deletions, additions, amino terminal truncations and carboxy-terminal truncations such that these mutations provide for proteins or protein fragments of diagnostic, therapeutic or prophylactic use and would be useful for screening for modulators, and/or inhibitors of MurF function.

Using the disclosure of polynucleotide and polypeptide sequences provided herein to isolate polynucleotides encoding naturally occurring forms of MurF, one of skill in the art can determine whether such naturally occurring forms are mutant or polymorphic forms of MurF by sequence comparison. One can further determine whether the encoded protein, or fragments of any MurF protein, is biologically active by routine testing of the protein of fragment in a *in vitro* or *in vivo* assay for the biological activity of the MurF protein. For example, one can express N-terminal or C-terminal truncations, or internal additions or deletions, in host cells and

test for their ability to catalyze the ATP-dependent addition of D-alanine-D-alanine to the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap precursor.

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that encode RNA comprising alternative codons which code for the eventual translation of the identical amino acid Therefore, the present invention discloses codon redundancy which can result in different DNA molecules encoding an identical protein. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide. However, any given change can be examined for any effect on biological function by simply assaying for the ability to catalyze the ATP-dependent addition of D-alanine-D-alanine to the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap precursor as compared to an unaltered MurF protein.

It is known that DNA sequences coding for a peptide can be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate.

As used herein, a "biologically active equivalent" or "functional derivative" of a wild-type MurF possesses a biological activity that is substantially similar to the biological activity of a wild type MurF. The term "functional derivative" is intended to include the "fragments," "mutants," "variants," "degenerate variants," "analogs," "orthologues," and "homologues" and "chemical derivatives" of a wild type MurF protein that can catalyze the ATP-dependent addition of D-alanine-D-alanine to the UDP-N-acetylmuramyl-L-alanine-D-Glutamine-m-Dap precursor. The term "fragment" refers to any polypeptide subset of wild-type MurF. The term "mutant" is meant to refer to a molecule that may be substantially similar to the wild-type form but possesses distinguishing biological characteristics. Such altered characteristics include but are in no way limited to altered substrate binding, altered substrate affinity and altered sensitivity to chemical compounds affecting biological activity of the MurF or MurF functional derivative. The term "variant" refers to a

molecule substantially similar in structure and function to either the entire wild-type protein or to a fragment thereof. A molecule is "substantially similar" to a wild-type MurF-like protein if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the exact structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the full-length MurF protein or to a biologically active fragment thereof.

As used herein in reference to a MurF gene or encoded protein, a "polymorphic" MurF is a MurF that is naturally found in the population of *Pseudomonads* at large. A polymorphic form of MurF can be encoded by a different nucleotide sequence from the particular *murF* gene disclosed herein as SEQ ID NO:1. However, because of silent mutations, a polymorphic *murF* gene can encode the same or different amino acid sequence as that disclosed herein. Further, some polymorphic forms MurF will exhibit biological characteristics that distinguish the form from wild-type MurF activity, in which case the polymorphic form is also a mutant.

A protein or fragment thereof is considered purified or isolated when it is obtained at least partially free from it's natural environment in a composition or purity not found in nature. It is preferred that the molecule be present at a concentration at least about five-fold to ten-fold higher than that found in nature. A protein or fragment thereof is considered substantially pure if it is obtained at a concentration of at least about 100-fold higher than that found in nature. A protein or fragment thereof is considered essentially pure if it is obtained at a concentration of at least about 1000-fold higher than that found in nature. We most prefer proteins that have been purified to homogeneity, that is, at least 10,000 -100,000 fold.

Probes and Primers

Polynucleotide probes comprising full length or partial sequences of SEQ ID NO: 1 can be used to determine whether a cell or sample contains *P. aeruginosa* MurF DNA or RNA. The effect of modulators that effect the transcription of the *murF* gene can be studied via the use of these probes. A preferred probe is a single stranded antisense probe having at least the full length of the coding sequence of *murF*. It is also preferred to use probes that have less than the full length sequence, and contain sequences specific for *P. aeruginosa murF* DNA or RNA. The

identification of a sequence(s) for use as a specific probe is well known in the art and involves choosing a sequence(s) that is unique to the target sequence, or is specific thereto. It is preferred that polynucleotides that are probes have at least about 25 nucleotides, more preferably about 30 to 35 nucleotides. The longer probes are believed to be more specific for *P. aeruginosa murF* gene(s) and RNAs and can be used under more stringent hybridization conditions. Longer probes can be used but can be more difficult to prepare synthetically, or can result in lower yields from a synthesis. Examples of sequences that are useful as probes or primers for *P. aeruginosa murF* gene(s) are Primer A (sense) 5'- TTTCATATGCTTGAGCCTCTTCGCCTC -3' (SEQ ID NO:3) and Primer B (antisense) 5'- TTGGATCCTTAGTGACTCTCCTCGGAG -3' (SEQ ID NO:4). These primers are nucleotides 1-21 (A) and the complement of nucleotides 1358-1376 (B) respectively, of SEQ ID NO:1. Restriction sites, underlined, for NdeI and BamHI are added to the 5' ends of the primers to allow cloning between the NdeI and BamHI sites of the expression vector pET-15b. However, one skilled in the art will recognize that these are only a few of the useful probe or primer sequences that can be derived from SEQ ID NO:1.

Polynucleotides having sequences that are unique or specific for *P. aeruginosa murF* can be used as primers in amplification reaction assays. These assays can be used in tissue typing as described herein. Additionally, amplification reactions employing primers derived from *P. aeruginosa murF* sequences can be used to obtain amplified *P. aeruginosa murF* DNA using the *murF* DNA of the cells as an initial template. The *murF* DNA so obtained can be a mutant or polymorphic form of *P. aeruginosa murF* that differs from SEQ ID NO:1 by one or more nucleotides of the *murF* open reading frame or sequences flanking the ORF. The differences can be associated with a non-defective naturally occurring form or with a defective form of MurF. Thus, polynucleotides of this invention can be used in identification of various polymorphic *P. aeruginosa murF* genes or the detection of an organism having a *P. aeruginosa murF* gene. Many types of amplification reactions are known in the art and include, without limitation, Polymerase Chain Reaction, Reverse Transcriptase Polymerase Chain Reaction, Strand Displacement Amplification and Self-Sustained Sequence Reaction. Any of these or like reactions can be used with primers derived from SEQ ID NO:1.

Expression of MurF

A variety of expression vectors can be used to express recombinant MurF in host cells. Expression vectors are defined herein as nucleic acid sequences that include regulatory sequences for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express a bacterial gene in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells and animal cells. Specifically designed vectors allow the shuttling of genes between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and regulatory sequences. A promoter is defined as a regulatory sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors can include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

In particular, a variety of bacterial expression vectors can be used to express recombinant MurF in bacterial cells. Commercially available bacterial expression vectors which are suitable for recombinant MurF expression include, but are not limited to pQE (Qiagen), pET11a or pET15b (Novagen), lambda gt11 (Invitrogen), and pKK223-3 (Pharmacia).

Alternatively, one can express *murF* DNA in cell-free transcription-translation systems, or *murF* RNA in cell-free translation systems. Cell-free synthesis of MurF can be in batch or continuous formats known in the art.

One can also synthesize MurF chemically, although this method is not preferred.

A variety of host cells can be employed with expression vectors to synthesize MurF protein. These can include *E. coli*, *Bacillus*, and *Salmonella*. Insect and yeast cells can also be appropriate.

Following expression of MurF in a host cell, MurF polypeptides can be recovered. Several protein purification procedures are available and suitable for use. MurF protein and polypeptides can be purified from cell lysates and extracts, or from culture medium, by various combinations of, or individual application of methods including ultrafiltration, acid extraction, alcohol precipitation, salt fractionation, ionic exchange chromatography, phosphocellulose chromatography, lecithin

chromatography, affinity (*e.g.*, antibody or His-Ni) chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and chromatography based on hydrophobic or hydrophillic interactions. In some instances, protein denaturation and refolding steps can be employed. High performance liquid chromatography (HPLC) and reversed phase HPLC can also be useful. Dialysis can be used to adjust the final buffer composition.

The MurF protein itself is useful in assays to identify compounds that modulate the activity of the protein -- including compounds that inhibit the activity of the protein. The MurF protein is also useful for the generation of antibodies against the protein, structural studies of the protein, and structure/function relationships of the protein.

Modulators and Inhibitors of MurF

The present invention is also directed to methods for screening for compounds which modulate or inhibit a MurF protein. Compounds which modulate or inhibit MurF can be DNA, RNA, peptides, proteins, or non-proteinaceous organic or inorganic compounds or other types of molecules. Compounds that modulate the expression of DNA or RNA encoding MurF or are inhibitors of the biological function of MurF can be detected by a variety of assays. The assay can be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay can be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample, that is, a control. A compound that is a modulator can be detected by measuring the amount of the MurF produced in the presence of the compound. An compound that is an inhibitor can be detected by measuring the specific activity of the MurF protein in the presence and absence of the compound.

The proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and analysis of MurF. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant MurF or anti- MurF antibodies suitable for detecting MurF. The carrier can also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutical Compositions

Pharmaceutically useful compositions comprising a modulator or inhibitor of MurF can be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation can be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the inhibitor.

Therapeutic, prophylactic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat, prevent or diagnose disorders. The effective amount can vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician

The pharmaceutical compositions can be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties can improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties can attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The present invention also provides a means to obtain suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The following examples are presented by the way of illustration and, because various other embodiments will be apparent to those in the art, the following is not to be construed as a limitation on the scope of the invention. For example, while particular preferred embodiments of the invention are presented herein, it is within the ability of persons of ordinary skill in the art to modify or substitute vectors, host cells, compositions, etc., or to modify or design protocols or assays, all of which may reach the same or equivalent performance or results as the embodiments shown herein.

EXAMPLE 1

General Materials and Methods

All reagents were purchased from SIGMA CHEMICAL CO., St. Louis, MO, unless otherwise indicated. UDP-N-acetylmuramyl-L-alanine was synthesized and purified by a method known in the art (Jin, H., Emanuele, J. J., Jr., Fairman, R., Robertson, J. G., Hail, M. E., Ho, H.-T., Falk, P. and Villafranca, J. J, 1996. Structural studies of *Escherichia coli* UDP-N-acetylmuramate: L-alanine ligase, Biochemistry 35: 14423-14431).

DNA manipulations reagents and techniques.

Restriction endonucleases and T4 ligase were obtained from Gibco-BRL. Agarose gel electrophoresis and plasmid DNA preparations were performed according to published procedures (Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, Molecular cloning: a L, Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). Recombinant plasmids containing *P. aeruginosa murF* were propagated in *E. coli* DH5a (GIBCO-BRL, Rockville, MD) prior to protein expression in *E. coli* BL21(DE3)/plysS (NOVAGEN, Madison, WI). SDS-PAGE was performed with precast gels (NOVAGEN). DNA sequences were determined using an automated ABI PRISM^{TM} DNA sequencer (PERKIN-ELMER ABI, Foster City, CA).

EXAMPLE 2

Cloning of Pseudomonas aeruginosa murF

Genomic DNA from *P. aeruginosa* (strain MB4439) was prepared from 100 ml late stationary phase culture in Brain Heart Infusion broth (DIFCO, Detroit, MI). Cells were washed with 0.2 M sodium acetate, suspended in 10 ml of TEG (100 mM Tris, pH 7, containing 10 mM EDTA and 25% glucose) and lysed by incubation with 200 μg of N-acetylmuramidase (SIGMA) for 1h at 37°C. Chromosomal DNA was purified from the cell lysate using a QIAGEN (Santa Clarita, CA) genomic DNA preparation kit and following the manufacturers protocol. Briefly, the cell lysate was treated with protease K at 50°C for 45 min, loaded onto an

equilibrated QIAGEN genomic tip, entered into the resin by centrifugation at 3000 rpm for 2 min. Following washing the genomic tip, the genomic DNA was eluted in distilled water and kept at 4°C. Approximately 50 ng genomic DNA was used as a template in PCR reactions to clone *murF*.

Two oligonucleotide primers (GIBCO/BRL, Bethesda, MD) complementary to sequences at the 5' and the 3' ends of *P. aeruginosa murF* were used to clone this gene using KLENTAQ ADVANTAGE™ polymerase (CLONTECH, Palo Alto, CA). The primer nucleotide sequences were as follows: 5'- TTTCATATGCTTGAGCCTCTTCGCCTC -3' (SEQ ID NO:3) (a NdeI linker plus nucleotides 1-21 of SEQ ID NO: 1) and 5'- TTGGATCCTTAGTGACTCTCCTCGGAG -3' (SEQ ID NO:4) (a BamH1 linker plus the complement of nucleotides 1358-1376 of SEQ ID NO: 1). A PCR product representing *P. aeruginosa murF* was verified by nucleotide sequence, digested with NdeI and BamHI, and cloned between the NdeI and BamHI sites of pET-15b, creating plasmid pPaeMurF. This plasmid was used for expression of the *murF* gene in *E. coli*.

The plasmid pPaeMurF has been deposited with the American Type Culture Collection on_____, 1999, under the terms of the Budapest Treaty for the Deposit of Microorganisms and has been designated as ATCC_____. The deposited material is provided as a convenience and is not an indication that the deposited material is required to describe or practice the invention. The sequence of the polynucleotide of the deposit, and the encoded amino acid sequence, are incorporated herein by reference and are controlling in the event of a conflict with any description of the sequences provided in this specification or the associated drawings. A license may be required to make, use, sell or offer to sell the polynucleotide of the deposit or a protein of the amino acid sequence encoded by the polynucleotide. No such license is granted herein.

EXAMPLE 3

Sequence analysis of Pseudomonas aeruginosa murF

The nucleotide sequence of *murF*, determined in both orientations, and the deduced amino acid sequence of the MurF protein is depicted in FIG. 1. Sequence comparison using the BLAST (1) algorithm against the GenBank database showed

that, to varying degrees, the cloned region is homologous (62% similar, 44% identical) to *murF* gene from *E. coli* (Parquet, C., D., Mengin-Lecreulx, B. Flouret, D. Mengin-Lecreulx, and J. van Heijenoort, 1989. Nucleotide sequence of the murF gene encoding the UDP-MurNAc-pentapeptide synthetase of *Escherichia coli.*, Nucleic Acids Res. 17:5379).

EXAMPLE 4

Overexpression, purification and enzymatic activity of *Pseudomonas aeruginosa* MurF

murF was cloned into the expression vector pET-15b (Novagen) as described above to create plasmid pPaeMurF. The pET-15b vector incorporates the 6xHistidine-tag into the protein construct to allow rapid purification of MurF by affinity chromatography. The pET (Plasmids for Expression by T7 RNA polymerase) plasmids are derived from pBR322 and designed for protein over-production in *E. coli*. The vector pET-15b contains the ampicillin resistance gene, ColE1 origin of replication in addition to T7 phage promoter and terminator. The T7 promoter is recognized by the phage T7 RNA polymerase but not by the *E. coli* RNA polymerase. A host *E coli* strain such as BL21(DE3)pLysS is engineered to contain integrated copies of T7 RNA polymerase under the control of lacUV5 that is inducible by IPTG. Production of a recombinant protein in the *E. coli* strain BL21(DE3)pLysS occurs after expression of T7RNA polymerase is induced.

The pPaeMurF plasmid was introduced into the host strain BL21 DE3/pLysS (NOVAGEN) for expression of His-tagged MurF. Colonies were grown at 37°C in 100 ml of LB broth containing 100 mg/ml ampicillin and 32 μg/ml chloramphenicol. When cultures reached a cell density of A_{600}=0.5, cells were pelleted and then resuspended in M9ZB medium (NOVAGEN) containing 1 mM IPTG. Cells were induced for 3 h at 30°C, pelleted at 3000*g*, and frozen at -80°C.

Cultures containing either the recombinant plasmid pPaeMurF or the control plasmid vector, pET-15b were grown at 30°C and induced with IPTG. Cells transformed with pPaeMurF contained an inducible protein of approximately 51.6 kDa, corresponding to the expected size of *P. aeruginosa* MurF protein as shown by SDS-PAGE. There were no comparable detectable protein bands after induction of cells transformed with the control plasmid vector, pET-15b.

Purification of recombinant MurF enzyme.

The cell pellet from 100 ml of induced culture prepared as described above was resuspended in 10 ml BT buffer (50 mM bis-tris-propane, pH 8.0, containing 100 mM potassium chloride and 1% glycerol) at 4°C. Cells were lysed either by freeze-thaw or by French Press. After centrifugation, the supernatant was mixed with 15 ml of freshly prepared TALON (CLONTECH) resin and incubated for 30 min at room temp. The resin was washed twice by centrifugation with 25 ml of BT buffer at room temperature. Finally, the resin was loaded into a column and washed with 20 ml of BT, pH 7.0, containing 5 mM imidazole. Protein was eluted with 20 ml of BT buffer pH 8.0, containing 100 mM imidazole. Fractions (0.5 ml) were collected and analyzed by SDS-Gel electrophoresis. This resulted in a partially purified preparation of *P. aeruginosa* MurF protein that could be used in activity assays. The protein may be purified further, if desired, using methods known in the art.

The *P. aeruginosa murF* was cloned into pET-15b between the NdeI and the BamHI sites and expressed in *E. coli* strain BL21(DE3)/pLysS. The recombinant MurF enzyme was affinity purified and eluted in 100 mM imidazole. Aliquots from cell lysates, either uninduced or induced with IPTG, and column-purified polypeptides were analyzed by SDS-PAGE (FIG. 2).

Assay for activity of MurF enzyme.

The ATP-dependent MurF activity was assayed by monitoring the formation of product ADP using the pyruvate kinase and lactate dehydrogenase coupled enzyme assay. The reaction was monitored spectrophotometrically.

Typically, the assay contained 100 mM BIS-TRIS-propane, pH 8.0, 200 μM NADH, 1 mM ATP, 20 mM PEP, 5 mM $MgCl_2$, 1 mM DTT, 350 μM UDP-N-acetyl-muramyl-L-alanine-D-Glutamine-m-Dap, 1 mM D-alanine-D-alanine, 33 units/ml of pyruvate kinase and 1660 units/ml of lactate dehydrogenase in a final volume of 200 or 400 μl. The mixture was incubated at 25°C for 5 min and the reaction initiated by the addition of 1-10 μg of MurF. These conditions are one example of an assay useful for evaluating the activity of MurF. Other assays can be used, or amounts of buffers, substrate and enzyme can be changed, as desired, to alter the rate of production of ADP.

ADP formation was monitored by the decrease in absorbance at 340 nm as a function of time using a SPECTRAMAXPLUS (MOLECULAR DEVICES) microtiterplate spectrophotomer (for 200 μl assays) or a HEWLETT-PACKARD

HP8452A spectrophotometer equipped with a circulating water bath (for 400 μl assays). Rates were calculated from the linear portions of the progress curves using the extinction coefficient for NADH, e = 6220 cm^{-1} M^{-1}. One unit of MurF activity is equal to 1 μmol of ADP formed per min at 25°C. MurF activity co-eluted with a ~51 kDa protein.

Table 1
Specific activities of recombinant MurF
from *E. coli* and *P. aeruginosa*.

Mur Ligase	*P. aeruginosa* μmol x min^{-1} x mg^{-1}	*E. coli* μmol x min^{-1} x mg^{-1}
MurF	3.41	1.15

EXAMPLE 5

Screening for inhibitors of MurF

One assay for the measurement of the activity of MurF is provided in Example 4. That assay, and other assays for MurF activity can be adapted for screening assays to detect inhibitors of MurF. For example, for inhibition assays, inhibitors in DMSO are added at the desired concentration to the assay mixture. In a separate, control reaction, only DMSO is added to the assay mixture. The reactions are initiated by the addition of enzyme (MurF). Rates are calculated as described above. Relative activities are calculated from the equation 1:

relative activity = rate with inhibitor/rate without inhibitor. (1)

Inhibition constant (IC_{50}) values are determined from a range of inhibitor concentrations and calculated from equation 2.

relative activity= $1/(1 + [I]/IC_{50})$ (2)

One can use computer software to assist in the analysis, *e.g.*, SIGMA PLOT™ (JANDEL SCIENTIFIC, San Rafeal).

We prefer inhibitors of MurF that result in relative activities of the MurF enzyme of at least less than 75%, more preferably, 25-50% or 10-25%. We most prefer inhibitors resulting in relative activities of less than 20%, particularly less than 10% of the activity of MurF in the absence of the inhibitor.

We also prefer inhibitors that effectively lower the relative activity of MurF when the inhibitor is present at a very low concentration.

EXAMPLE 8

Therapy using inhibitors of MurF

A patient presenting with an indication of infection with a microorganism susceptible to inhibitors of MurF, *e.g.*, gram positive and negative bacteria, including *P. aeruginosa*, can be treated by administration of inhibitors of MurF. Physicians skilled in the art are familiar with administering therapeutically effective amounts of inhibitors or modulators of microbial enzymes. Such skilled persons can readily determine an appropriate dosing scheme to achieve a desired therapeutic effect.

Therapy can also be prophylactic. For example, a patient at risk for developing a bacterial infection, including infection with *P. aeruginosa*, can be treated by administration of inhibitors of MurF. Physicians skilled in the art are familiar with administering therapeutically effective amounts of inhibitors or modulators of microbial enzymes. Such skilled persons can readily determine an appropriate dosing scheme to achieve a desired therapeutic effect.

WHAT IS CLAIMED:

1. A purified and isolated polynucleotide selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(b) a polynucleotide which is complementary to the polynucleotide of (a),

(c) a polynucleotide representing a naturally occurring mutant or polymorphic form of (a),

(d) a polynucleotide that hybridizes with a polynucleotide of (a), (b), or (c) under stringent conditions, and

(e) a polynucleotide comprising at least 25 nucleotides of the polynucleotide of (a), (b) or (c), said 25 nucleotides being specific for *murF* gene of *Pseudomonas aeruginosa*.

2. The polynucleotide of claim 1 wherein the polynucleotide comprises nucleotides selected from the group consisting of natural, non-natural and modified nucleotides.

3. The polynucleotide of claim 1 wherein the internucleotide linkages are selected from the group consisting of natural and non-natural linkages.

4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1.

5. A polynucleotide that is an expression vector comprising a polynucleotide of claim 1.

6. A host cell comprising the expression vector of claim 5.

7. A process for expressing a MurF protein of *Pseudomonas aeruginosa* in a recombinant host cell, comprising:

(a) transforming a suitable host cell with an expression vector of claim 5; and,

(b) culturing the host cell of step (a) in conditions under which allow expression of said the MurF protein from said expression vector.

8. A purified and isolated polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

9. A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing at least one host cell harboring an expression vector that includes a polynucleotide selected from the group consisting of:

(i) a polynucleotide encoding a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(ii) a polynucleotide which is complementary to the polynucleotide of (i),

(iii) a polynucleotide representing a naturally occurring mutant or polymorphic form of (i), and

(b) contacting at least one of said cells with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the polypeptide in the presence of the candidate.

10. The method of claim 9 wherein the polynucleotide has the nucleotide sequence of SEQ ID NO:1.

11. The method of claim 9 wherein in step (c) the relative activity is determined by comparing a measurement of MurF polypeptide activity of at least one cell before step (b) to a measurement of MurF polypeptide activity of at least one cell after step (b).

12. A compound that is an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

13. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide that is a naturally occurring mutant or polymorphic form of (a).

14. A method of treatment of a patient in need of prophylactic or therapeutic treatment for a bacterial infection comprising administering to the patient an effective amount of an inhibitor of a polypeptide having an amino acid sequence selected from the group consisting of

(a) a polypeptide having an amino acid sequence of SEQ ID NO:2,

(b) a polypeptide representing a naturally occurring mutant or polymorphic form of (a).

15. A method of determining whether a candidate compound is an inhibitor of a *Pseudomonas aeruginosa* MurF polypeptide comprising:

(a) providing a sample that includes a MurF polypeptide selected from the group consisting of:

(i) a polypeptide having an amino acid sequence of SEQ ID NO: 2.

(ii) a polypeptide that is a functional derivative of the polypeptide of (i),

(iii) a polypeptide representing a naturally occurring mutant or polymorphic form of (i), and

(b) contacting said sample with the candidate to permit the interaction of the candidate with the MurF polypeptide, and

(c) determining whether the candidate is an inhibitor of the MurF polypeptide by ascertaining the relative activity of the MurF polypeptide in the presence of the candidate.

16. The method of claim 15 wherein the polypeptide has the amino acid sequence of SEQ ID NO:2.

17. The method of claim 15 wherein in step (c) the relative activity is determined by comparing a measurement of MurF polypeptide activity of the sample before step (b) to a measurement of MurF polypeptide activity of the sample after step (b).

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(54) Title: MURF GENE AND ENZYME OF *PSEUDOMONAS AERUGINOSA*

(57) Abstract: This invention provides isolated polynucleotides that encode the MurF protein of *Pseudomonas aeruginosa*. Purified and isolated MurF recombinant proteins are also provided. Nucleic acid sequences which encode functionally active MurF proteins are described. Assays for the identification of modulators of the expression of *mur* and inhibitors of the activity of MurF, are also provided.

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35 40 45
Phe Asp Gly His Asp Tyr Leu Ala Glu Val Ala Ala Lys Gly Ala Val

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Arg Arg Lys Phe Thr Gly Pro Leu Ala Ala Met Thr Gly Ser Ser Gly
            100                 105                 110
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                165                 170                 175
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            180                 185                 190
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        195                 200                 205
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225                 230                 235                 240
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305                 310                 315                 320
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                325                 330                 335
Asp Asp Ser Tyr Asn Ala Asn Pro Ala Ser Met Leu Ala Ala Ile Asp
            340                 345                 350
Ile Leu Ser Gly Phe Ser Gly Arg Thr Val Leu Val Leu Gly Asp Met
        355                 360                 365
Gly Glu Leu Gly Ser Trp Ala Glu Gln Ala His Arg Glu Val Gly Ala
    370                 375                 380
Tyr Ala Ala Gly Lys Val Ser Ala Leu Tyr Ala Val Gly Pro Leu Met
385                 390                 395                 400
Ala His Ala Val Gln Ala Phe Gly Ala Thr Gly Arg His Phe Ala Asp
                405                 410                 415
Gln Ala Ser Leu Ile Gly Ala Leu Ala Thr Glu Gln Pro Thr Thr Thr
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FIG. 1A

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  TACGAACTCGGAGAAGCGGAGTCGGTCAACTGCCAGCGCGACCTGCGGGCGGACTAGCCG
      ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
  MetLeuGluProLeuArgLeuSerGlnLeuThrValAlaLeuAspAlaArgLeuIleGly

61 GAGGACGCCGTCTTTTCGGCGGTTTCCACCGACAGTCGCGCCATCGGGCCCGGCCAACTG 120
   CTCCTGCGGCAGAAAAGCCGCCAAAGGTGGCTGTCAGCGCGGTAGCCCGGGCCGGTTGAC
       ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
   GluAspAlaValPheSerAlaValSerThrAspSerArgAlaIleGlyProGlyGlnLeu

121 TTCATTGCCCTGAGTGGGCCGCGTTTCGACGGCCACGACTATCTCGCCGAGGTTGCCGCC 180
    AAGTAACGGGACTCACCCGGCGCAAAGCTGCCGGTGCTGATAGAGCGGCTCCAACGGCGG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    PheIleAlaLeuSerGlyProArgPheAspGlyHisAspTyrLeuAlaGluValAlaAla

181 AAGGGCGCGGTGGCTGCGCTGGTGGAGCGCGAAGTCGCCGACGCGCCCTTGCCGCAATTG 240
    TTCCCGCGCCACCGACGCGACCACCTCGCGCTTCAGCGGCTGCGCGGGAACGGCGTTAAC
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    LysGlyAlaValAlaAlaLeuValGluArgGluValAlaAspAlaProLeuProGlnLeu

241 CTGGTGCGCGATACCCGTGCGGCCCTGGGGCGACTGGGCGCGCTGAACCGGCGCAAGTTC 300
    GACCACGCGCTATGGGCACGCCGGGACCCCGCTGACCCGCGCGACTTGGCCGCGTTCAAG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    LeuValArgAspThrArgAlaAlaLeuGlyArgLeuGlyAlaLeuAsnArgArgLysPhe

301 ACCGGCCCGCTGGCGGCCATGACGGGCTCCAGCGGCAAGACCGCGGTCAAGGAGATGCTC 360
    TGGCCGGGCGACCGCCGGTACTGCCCGAGGTCGCCGTTCTGGCGCCAGTTCCTCTACGAG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    ThrGlyProLeuAlaAlaMetThrGlySerSerGlyLysThrAlaValLysGluMetLeu

361 GCCAGCATCCTGCGTACCCAGGCCGGCGATGCCGAGTCGGTGCTGGCTACCCGTGGCAAT 420
    CGGTCGTAGGACGCATGGGTCCGGCCGCTACGGCTCAGCCACGACCGATGGGCACCGTTA
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    AlaSerIleLeuArgThrGlnAlaGlyAspAlaGluSerValLeuAlaThrArgGlyAsn

421 CTGAACAACGACCTCGGCGTACCGCTGACCCTGCTGCAACTGGCGCCGCAGCACCGTAGC 480
    GACTTGTTGCTGGAGCCGCATGGCGACTGGGACGACGTTGACCGCGGCGTCGTGGCATCG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    LeuAsnAsnAspLeuGlyValProLeuThrLeuLeuGlnLeuAlaProGlnHisArgSer

481 GCAGTGATCGAACTGGGCGCCTCGCGCATCGGCGAGATCGCCTACACGGTCGAGCTGACC 540
    CGTCACTAGCTTGACCCGCGGAGCGCGTAGCCGCTCTAGCGGATGTGCCAGCTCGACTGG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    AlaValIleGluLeuGlyAlaSerArgIleGlyGluIleAlaTyrThrValGluLeuThr
```

FIG. 1B

```
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    GCGGGCGTGCACCGCTAGTAGTGGTTACGGCCTTGGCGGGTACAGCCGCTCAAGCCGCCT
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    ArgProHisValAlaIleIleThrAsnAlaGlyThrAlaHisValGlyGluPheGlyGly

601 CCGGAGAAGATCGTCGAGGCGAAGGGCGAGATACTCGAAGGGCTGGCCGCCGACGGCACC 660
    GGCCTCTTCTAGCAGCTCCGCTTCCCGCTCTATGAGCTTCCCGACCGGCGGCTGCCGTGG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    ProGluLysIleValGluAlaLysGlyGluIleLeuGluGlyLeuAlaAlaAspGlyThr

660 GCCGTACTGAACCTGGACGACAAGGCCTTCGACACCTGGAAGGCCCGTGCCAGCGGCCGT 720
    CGGCATGACTTGGACCTGCTGTTCCGGAAGCTGTGGACCTTCCGGGCACGGTCGCCGGCA
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    AlaValLeuAsnLeuAspAspLysAlaPheAspThrTrpLysAlaArgAlaSerGlyArg

721 CCGTTGCTGACTTTCTCCCTCGACCGGCCCCAGGCCGATTTCCGCGCCGCCGATCTGCAG 780
    GGCAACGACTGAAAGAGGGAGCTGGCCGGGGTCCGGCTAAAGGCGCGGCGGCTAGACGTC
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    ProLeuLeuThrPheSerLeuAspArgProGlnAlaAspPheArgAlaAlaAspLeuGln

781 CGCGATGCGCGCGGCTGCATGGGCTTCAGGCTGCAGGGCGTAGCGGGTGAAGCGCAGGTC 840
    GCGCTACGCGCGCCGACGTACCCGAAGTCCGACGTCCCGCATCGCCCACTTCGCGTCCAG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    ArgAspAlaArgGlyCysMetGlyPheArgLeuGlnGlyValAlaGlyGluAlaGlnVal

841 CAGCTCAACCTGCTGGGGCGGCACAATGTCGCCAATGCCCTGGCTGCGGCCGCTGCCGCC 900
    GTCGAGTTGGACGACCCCGCCGTGTTACAGCGGTTACGGGACCGACGCCGGCGACGGCGG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    GlnLeuAsnLeuLeuGlyArgHisAsnValAlaAsnAlaLeuAlaAlaAlaAlaAlaAla

901 CATGCACTGGGCGTGCCGCTGGATGGGATCGTCGCCGGGCTGCAGGCGCTGCAGCCGGTC 960
    GTACGTGACCCGCACGGCGACCTACCCTAGCAGCGGCCCGACGTCCGCGACGTCGGCCAG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    HisAlaLeuGlyValProLeuAspGlyIleValAlaGlyLeuGlnAlaLeuGlnProVal

961 AAGGGCCGCGCGGTAGCGCAACTGACCGCCAGCGGGCTGCGTGTGATAGACGACAGCTAC 1020
    TTCCCGGCGCGCCATCGCGTTGACTGGCGGTCGCCCGACGCACACTATCTGCTGTCGATG
        ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
    LysGlyArgAlaValAlaGlnLeuThrAlaSerGlyLeuArgValIleAspAspSerTyr

1021 AACGCCAACCCCGCGTCAATGCTGGCGGCGATTGATATACTGAGCGGCTTTTCCGGGCGC 1080
     TTGCGGTTGGGGCGCAGTTACGACCGCCGCTAACTATATGACTCGCCGAAAAGGCCCGCG
         ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
     AsnAlaAsnProAlaSerMetLeuAlaAlaIleAspIleLeuSerGlyPheSerGlyArg
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FIG. 1C

```
1081 ACCGTCCTGGTCCTCGGAGACATGGGCGAACTCGGTTCCTGGGCCGAGCAGGCCCACCGC 1140
     TGGCAGGACCAGGAGCCTCTGTACCCGCTTGAGCCAAGGACCCGGCTCGTCCGGGTGGCG
         ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
     ThrValLeuValLeuGlyAspMetGlyGluLeuGlySerTrpAlaGluGlnAlaHisArg

1141 GAGGTGGGCGCCTACGCCGCTGGCAAGGTGTCCGCGCTCTATGCGGTCGGACCGCTGATG 1200
     CTCCACCCGCGGATGCGGCGACCGTTCCACAGGCGCGAGATACGCCAGCCTGGCGACTAC
         ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
     GluValGlyAlaTyrAlaAlaGlyLysValSerAlaLeuTyrAlaValGlyProLeuMet

1201 GCCCACGCCGTACAGGCGTTCGGCGCCACGGGCCGGCACTTCGCCGACCAGGCCAGCCTG 1260
     CGGGTGCGGCATGTCCGCAAGCCGCGGTGCCCGGCCGTGAAGCGGCTGGTCCGGTCGGAC
         ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
     AlaHisAlaValGlnAlaPheGlyAlaThrGlyArgHisPheAlaAspGlnAlaSerLeu

1261 ATCGGGGCGCTGGCCACCGAACAACCGACAACCACCATTTTGATCAAGGGTTCCCGCAGT 1320
     TAGCCCCGCGACCGGTGGCTTGTTGGCTGTTGGTGGTAAAACTAGTTCCCAAGGGCGTCA
         ^   *   ^   *   ^   *   ^   *   ^   *   ^   *
     IleGlyAlaLeuAlaThrGluGlnProThrThrThrIleLeuIleLysGlySerArgSer

1321 GCGGCGATGGACAAAGTCGTCGCGGCGCTGTGCGGTTCCTCCGAGGAGAGTCACTAATGC 1380
     CGCCGCTACCTGTTTCAGCAGCGCCGCGACACGCCAAGGAGGCTCCTCTCAGTGATTACG
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1381 TCCTGCTGCTGGC 1440
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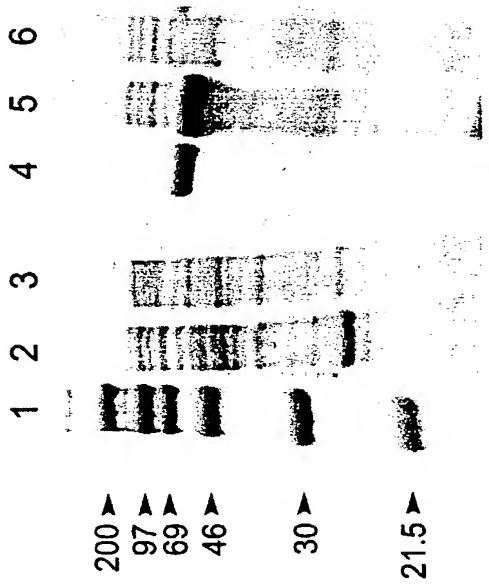
FIG. 2



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1007078 .030702

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SUBSTITUTE for PTO/SB/01 (12-97), DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

[X] Declaration Submitted with Initial Filing **OR** [ ] Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	20194P
First Named Inventor	MOHAMED EL-SHERBEINI ET AL.
COMPLETE IF KNOWN	
Application Number	TO BE ASSIGNED
Filing Date	MARCH 7, 2002
Group Art Unit	
Examiner Name	

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MURF GENE AND ENZYME OF PSEUDOMONAS AERUGINOSA

(Title of the Invention)

the specification of which

[X] is attached hereto

OR

[ ] was filed on (MM/DD/YYYY) [ ] as United States Application Number or PCT International Application Number [ ] and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Attorney Docket Number	Priority Claimed? YES	NO
PCT/US00/24437	PCT	09/06/2000	20194PCT	[ ]	[ ]
				[ ]	[ ]
				[ ]	[ ]
				[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	Attorney Docket Number
60/153,293	09/10/1999	20194PV

Please type a plus sign (+) inside this box → [+]

10070728.030702

Approved for use through 9/30/2000. OMB 651-0032

SUBSTITUTE for PTO/SB/01 (12-97), DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

DECLARATION AND POWER OF ATTORNEY for Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information known to me to be material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Application Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint, respectively and individually, as my attorneys or agents with full power of substitution and revocation, the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☐ Customer Number [ ] → *Place Customer Number Bar Code Label here*

OR

[X] Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
MICHAEL D. YABLONSKY	40,407	JACK L. TRIBBLE	32,633

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
MOHAMEND	EL-SHERBEINI

100

Inventor's Signature	Mohamed El-Sherbeini				**Date**	3/6/02	
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City	Rahway	**State**	NJ	**ZIP**	07065-0907		

[X] Additional inventors are being named on the 1 supplemental Additional Inventors(s) sheet(s) PTO/SB/02A attached hereto.

Please type a plus sign (+) inside this box → [+]

Approved for use through 9/30/2000. OMB 651-0032
SUBSTITUTE for PTO/SB/02A (3-97), Declaration (Additional Inventors)

DECLARATION AND POWER OF ATTORNEY

ADDITIONAL INVENTOR(S) Supplemental Sheet

200

Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
BARBARA				AZZOLINA			
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Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature					**Date**		
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Post Office Address	Merck & Co., Inc., P.O. Box 2000						
City	Rahway	**State**	NJ	**ZIP**	07065-0907		
Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature					**Date**		
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Name of Additional Joint Inventor, if any:		☐ A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any])				Family Name or Surname			
Inventor's Signature					**Date**		
Residence: City		**State**		**Country**		**Citizenship**	
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City	Rahway	**State**	NJ	**ZIP**	07065-0907		